Therapeutic effect of Ginsenoside Rb1 against mechanical trauma in a rat model of postpartum stress urinary incontinence

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Research Article

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Abstract

Objective

The aim of the present study was to investigate the therapeutic effect of ginsenoside Rb1 (GS-Rb1) on urethral mechanical trauma in a rat model of stress urinary incontinence (SUI) and the underlying mechanisms.

Design

24 female rats were divided into four groups: (1) control; (2) SUI; (3) SUI + L-Rb1; (4) SUI + H-Rb1 group. SUI models were induced via vaginal dilation (VD). Control rats received sham surgery.

Methods

The treatment rats received oral doses of ginsenoside Rb1 (4.5 mg/kg and 18 mg/kg, for the L and H groups, respectively) once per day for one week. Rats in the SUI group received the same volume of saline. On day 7, the leak point pressure (LPP) and maximum bladder capacity were evaluated and the rats were sacrificed. Urethral and vaginal wall tissue was examined using histology, immunohistochemistry, western blotting, and qPCR.

Results:

GS-Rb1 treatment was found to reduce urethral mechanical trauma, allowing periurethral tissue recovery. GS-Rb1 also reduced the numbers of fragmented and disorganized elastic and muscle fibers through activation of Nrf2/ARE and TGF-β1/Smad3, leading to increased collagen synthesis and ameliorating damage to the urethral fibromuscular system.

Conclusion:

GS-Rb1 effectively reduced mechanical trauma to the urethral fibromuscular system in an SUI rat model. This was mediated by activation of Nrf2 antioxidant activity and TGF-β1/Smad3 signaling.

Introduction

Stress urinary incontinence (SUI) is a urological disorder often found in women after childbirth and involves involuntary urine loss when pressure on the abdomen is increased, as during coughing or exercise[1].

The normal function of the female pelvic floor requires an intact anatomical structure[2]. Mechanical trauma damage to its suspensory structures, the defects of weakening periurethral connective tissues in the periurethral ligament and periurethral ligament disruption lead to a reduction in urethral closure pressure and are significantly associated with SUI. Histological findings in periurethral tissues of women
with SUI consistently show abnormal collagen remodeling and loss of functional elastic fiber networks and muscle fiber rupture[3].

Vaginal delivery-induced pelvic tissue mechanical trauma is the primary etiological factor of SUI[4]. At present, some therapy methods for SUI, such as periurethral injection of bulking agents, stem cells injection and pelvic floor function exercise, cannot provided an ideal long-term outcomes[5-7]. Various sling surgeries are more efficacious and prevalent but are associated with serious complications, such as urinary tract injury and infection[8]. Currently, there are few studies on the repair of trauma caused by childbirth from a pathological perspective in the early postpartum period.

Injury-associated remodeling of the extracellular matrix (ECM) is closely associated with both SUI and pelvic organ prolapse (POP)[9, 10]. Transforming growth factor β1 (TGF-β1) modulates ECM structure through regulation of fibronectin, collagen, and elastin through phosphorylation of Smad2 and Smad3[9, 10]. There is evidence that TGF-β1/Smad mediated remodeling of the ECM is associated with SUI pathology.

SUI is also associated with oxidative damage resulting from mechanical trauma[9, 11]. Studies using SUI rat models have demonstrated the promotion of both oxidative injury and apoptosis by vaginal trauma[3]. The transcription factor nuclear factor-E2-related factor 2 (Nrf2) responds to oxidative stress by interacting with antioxidant response elements (AREs) to promote the expression of a variety of antioxidant genes[12, 13]. Nrf2 has been shown to protect against apoptosis resulting from mechanical stretching in mouse fibroblasts and suggests that it may be involved in SUI pathogenesis also. It is also possible that ROS may function as cellular messengers to regulate intracellular signaling, including the TGF-β1/Smad pathway[14, 15].

Ginsenoside Rb1(GS-Rb1) is an active ingredient of *Panax ginseng*, *Notoginseng radix* with antioxidant and anti-inflammatory activities, as well as the ability to regulate autophagy, lipid, and sugar metabolism, reduce apoptosis, and influence cytokine production[12, 13, 16]. GS-Rb1 has also been shown to modulate the expression and activity of numerous proteins and pathways, including the TGF-β1/Smad, PI3K/mTOR, Nrf2/ARE, and MAPK/NF-κb pathways[17]. It is feasible that GS-Rb1 may mitigate oxidative damage and stimulate matrix and tissue repair in SUI.

In the present study, we explored the role of GS-Rb1 in treating urethral mechanical trauma using an SUI rat model and investigated that the underlying mechanisms involved Nrf2 and TGF-β1/Smad3 signal.

**Materials And Methods**

**SUI Model and experimental design**

Twenty-four Sprague-Dawley rats (female, two months old; 236.4±15.2 g) were obtained from the animal experimental center of Shanxi Medical University. The rats were maintained in cages of six at 25°C±2°C and a 12-h light/dark cycle, and ad libitum food and water. All animal procedures were performed by
following the Guide for the Care and Use of Laboratory Animals and approved by the Animal Experimental Ethics Committee of Shanxi Cancer Hospital (Approval Number:2021001, Shanxi, China). GS-Rb1 was obtained from the China Yunnan Teana Pharmaceutical Co. LTD (Yunnan, China). Four groups were established (n = 6 per group): control; SUI; SUI+ H-Rb1; SUI+ L-Rb1. Control rats received sham surgery. The animals were anesthetized by intraperitoneal administration of 10% chloral hydrate (3 mL/kg) and placed on their backs. A balloon containing 3 mL of saline was inserted into the vagina with a transurethral 12F catheter and left in place for 3 h. Dysfunctional voiding was apparent in all rats following the procedure. GS-Rb1 (4.5 mg/kg and 18mg/kg in saline) was administered intragastrically to the SUI+GS-Rb1 group after 24 h and thereafter for one week. Rats in the SUI only group received the same volume of saline. Rats were assessed for urinary function and sacrificed 24 h after drug withdrawal and urethral tissue was collected for further analysis.

Assessment of urodynamics

Rats were anesthetized as above and intravesicular catheterization was performed via suprapubic cystostomy using a PE-50 polyethylene tube with a flared end as an anchor. Saline was introduced with a 50-mL syringe. The LPP was determined from the syringe elevation. Bladder capacities were measured after 3–5 voiding cycles. At the point of 50% bladder capacity, a finger was placed on the animal’s abdomen and pushed gently until urine leaked. The maximum pressure used was defined as the LPP. This was performed three times on each rat and the average LPP was determined. After sacrifice, urethral and anterior vaginal walls were harvested.

Histological and immunohistochemical analyses

Tissues were fixed with 10% formalin, paraffin-embedded, and sectioned. Masson’s trichrome and Picosirius red stains were applied for collagen detection and Hart’s stain for elastin using standard protocols. Immunohistochemistry was performed according to a protocol (BIOS Biology Co., Ltd, China). Image Pro Plus 5.1 was used to determine staining intensities.

RT-PCR

Total RNA was extracted using TRIzol (HaDa Biotech, Taiyuan, China) and reverse-transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (HaDa Biotech). Quantitative PCR was performed on a Real-Time PCR platform (Applied HaDa Biotech) following provided directions. GADPH was used for normalization and relative expression was determined by the $2^{-\Delta\Delta Ct}$ method. Primer sequences in Real-Time PCT are provided in additional file 1 [see Additional file 1].

Protein extraction and western blotting

The tissue was homogenized in RIPA lysis buffer. Twenty micrograms of protein per lane were separated on 10% SDS-PAGE and transferred to PVDF (Biosis, Beijing, China). After blocking, the blots were sequentially probed with primary and secondary antibodies (1:10000). The antibodies used were anti-
collagen I (bs-10423R) and anti-collagen III (bs-0549R)(Bioss, Beijing, China), anti-TGF-β1 (BD-PT4632), anti-Smad2 (BD-PT4331), anti-Smad3 (BD-PT4334), anti-Smad7 (BD-PN2330), anti-p-Smad2 (BD-PP0584), anti-p-Smad3 (BD-PP1501), anti-Nrf2 (BD-PT3189), anti-SOD-2 (BD-PT5575), anti-GPX-1 (BD-PN2008) (Biodrgon, Beijing, China), and anti-β-Actin (Santa Cruz Biotechnology, CA, USA). Subsequently, the membranes were washed three times in TBST for 10 min each time, followed by incubation for 2 h with a goat anti-rabbit horseradish peroxidase-conjugated antibody (1:2000) and visualized via chemiluminescence. The quantification of bands was further analyzed by ImageJ software through grayscale module (n = 6 per group).

**Statistical analysis**

All data were expressed as means ± SD and analyzed using SPSS 16.0. Differences between groups were evaluated by one-way ANOVA, and P-values < 0.05 were considered significant.

**Results**

**GS-Rb1 promotes LPP and bladder capacity recovery after VD in L-Rb1 rats**

Cystometry, including LPP and bladder capacity measurement, was performed under anesthesia. Bladder capacities were greater in SUI rats (0.79 ± 0.19 ml) relative to controls (0.51 ± 0.05 ml) and SUI+L-Rb1 rats (0.55 ± 0.06 ml) but was reduced in SUI+H-Rb1 rats (0.41 ± 0.11 ml). The LPP was 33.9 ± 1.27 cm H2O in control rats and 24.5 ± 2.27 cm H2O in SUI model rats (Table. 1) (S Fig.1). Thus, the LPP was lower in SUI rats than in either the control and L-Rb1 rats additional file 2 [see Additional file 2].

| Cystometric parameters in four groups (x̄±s) |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                             | Control                       | SUI                          | SUI+L-Rb1                     | SUI+H-Rb1                     |
| LPP (cmH2O)                  | 33.9±1.27                     | 24.5±2.27 *                  | 29.9±1.15 #                  | 23.9±1.21 *                  |
| Bladder capacity (ml)        | 0.51±0.05                     | 0.79±0.19 *                  | 0.55±0.06 #                  | 0.41±0.11 #                  |

LPP values of rats in SUI group and H-Rb1 group were significantly lower than the control group, whereas, values were similar in L-Rb1 group compared with the control group. *P <0.05 vs control group; #P <0.05 vs SUI group. Every measurement was repeated for tree times.

**VD:** vaginal distention, **Rb1:** Ginsenoside Rb1, **LPP:** leak-point pressure.

**Histological changes in the fibromuscular system**

While control rats showed compact and circumferential urethral striated muscle on Masson staining, marked changes, specifically, the splitting of muscle bundles, were visible in tissue from SUI rats. The muscles of the urethral sphincter in the GS-Rb1-treated groups were, however, similar to the controls. In addition, the collagen contents of tissues from the SUI rats were reduced compared with the GS-Rb1
treated rats (Fig. 1) [see Additional file 3]. Picosirius red staining indicated that the vaginal wall became thinner in SUI group and the content of collagen in the urethras and vaginal wall tissue of SUI rats were reduced in comparison with the other groups (Fig. 1). The percentage of collagens in the urethral and anterior vaginal wall tissue in the SUI group was significantly decreased than other groups [see Additional file 3]. Elastic fibers were abundant in smooth muscle from control rats with organization, and tight connection to the muscle bundles of smooth muscle and also lined up in the vaginal wall tissue, whereas, in SUI rats, the fibers appeared fragmented and disorganized in the muscle and vaginal wall tissue (Fig. 2). However, in the GS-Rb1-treated animals, morphological distribution of elastic fibers were markedly similar in appearance to those of the control rats.

**GS-Rb1 stimulates TGF-β1/Smad3 and promotes ECM recovery**

The part played by TGF-β1/Smad3 in recovery from trauma induced by VD was investigated. We profiled gene expression of ECM recovery in the tissue surrounding the urethra and the vaginal wall. Compared to SUI group, GS-Rb1 resulted in a significant increase in mRNA levels of the TGF-β1 and Smad3, as well as those of collagen I and III Fig. 3A. Western Blot results further confirmed that the protein levels of the TGF-β1 and Smad3, collagen I and III, were also increased in GS-Rb1-treated groups (Fig. 3B, 3C). TGF-β1 is known to be involved in injury repair processes. Mechanical trauma caused by VD was significantly inhibited by 4.5 and 18 mg/kg GS-Rb1 treatment. This indicates that GS-Rb1 treatment stimulated TGF-β1/Smad3 signaling and promoted recovery of the ECM.

**GS-Rb1 mitigates oxidative damage in SUI rats by stimulating Nrf2/ARE**

GS-Rb1 protected and reduced damage to urethral tissue and allowed structural recovery. E2-related factor 2 (Nrf2) responded to oxidative stress by interacting with antioxidant response elements (AREs) to promote the expression of a variety of antioxidant genes. Compared with the SUI group, in 4.5 and 18 mg/kg GS-Rb1 treatment groups, the structure of the periurethral muscles remained intact, with no significant breakage of the elastic fibers. GS-Rb1 resulted in a significant increase in protein levels of the Nrf2, GPX1, and MnSOD Fig. 4A. In H-Rb1 group, the levels of those protein were higher than L-Rb1 group (Fig. 4B,C,D). This indicates that GS-Rb1 treatment may be associated with stimulation of Nrf2/ARE signaling and inhibition of further tissue damage after mechanical trauma after VD.

**Discussion**

The principal etiological factor for SUI is mechanical trauma to the pelvic tissue resulting from vaginal delivery during childbirth[18]. The chronic damage to the sphincter muscle and its associated nerve supply tends to progress over time[19], indicating that these injuries do not fully heal. Postpartum SUI is common and, in many cases, does not resolve within three months[20]. In addition, many patients who recover may be at risk of SUI at a later stage. Thus, timely treatment may both mitigate postpartum SUI and reduce the chances of further development. Here, we investigated the effects of GS-Rb1 on SUI rat model, finding that it was able to mitigate the effects of trauma on the urethra and surrounding tissue.
It was found that the urethral microstructure recovered significantly better with GS-Rb1 treatment relative to rats with untreated SUI. SUI model rats showed visible disruption of the urethral muscle fibers, together with reduced connective tissue and collagen expression. GS-Rb1 mitigated damage to the muscle fibers and increased collagen concentrations. This indicates that GS-Rb1 can stimulate recovery from SUI induced by mechanical trauma, as well as increase collagen production and restore the thickness and length of the collagen fibers.

Elastic fibers in the ECM promote tissue flexibility and are necessary for normal urethral functioning[21]. Here, GS-Rb1 treatment was found to protect against injury to the elastic fibers in both the urethral and vaginal striated muscle.

To evaluate the effects of GS-Rb1 on SUI, urodynamic studies were conducted on living rats, assessing the LPP and bladder capacity. Both these parameters were adversely affected by SUI but were improved in the SUI+L-Rb1 group, indicating that L-Rb1 effectively protected urinary function. However, the urodynamic results in the H-Rb1+SUI rats appeared inconsistent with the histopathology of the urethral tissue in this group, as both LPP and bladder capacity were reduced. It is possible that H-Rb1 stimulates the growth of muscle fibers and the deposition of collagen, which increases the intravesical pressure to a level that exceeds the urethral closure pressure. Thus, after assessing both the histological and functional effects, an GS-Rb1 dose of 4.5 mg/kg/day was considered feasible for SUI rats where GS-Rb1 has effective therapeutic action but minimal adverse effects.

TGF-β1 is known to be involved in injury repair processes, together with its functions in cellular proliferation, differentiation, and survival[21, 22]. It is specifically involved in the regulation of the ECM, where it phosphorylates Smad2 and Smad3 to stimulate the expression of the ECM components collagen, fibronectin, and elastin[23, 24]. Involvement of TGF-β1 has also been demonstrated in SUI pathogenesis resulting from mechanical trauma[14, 25]and has been suggested as a target for SUI treatment[24, 26]. Thus, we evaluated the effects of GS-Rb1 on TGF-β1 and its associated proteins. It was found that SUI rats showed reduced levels of TGF-β1, Smad3, p-Smad3, and collagens I and III relative to both the control and GS-Rb1-treated rats. GS-Rb1 thus restored the deficits of TGF-β1/Smad3 signaling resulting from VD, promoting recovery of myocytes and collagen I and III synthesis.

Nrf2 modulates cell responses to oxidative stress by promoting the transcription of antioxidant genes carrying AREs in their promoter regions[24, 26]. It has been found that mechanical trauma induces oxidative damage to both the urethral sphincter and the vaginal wall, often leading to pelvic floor dysfunction (PFD)[25, 27]. Addressing oxidative damage may be critical for the prevention and treatment of SUI[7, 10, 25]. Here, the primary findings are the demonstration of Nrf2/ARE activation after GS-Rb1 treatment of SUI, with GS-Rb1 reducing both tissue injury. Nrf2 protein levels were also assessed by western blotting. This showed increased expression of Nrf2 in the SUI+ GS-Rb1 rats compared with rats that were not treated with GS-Rb1, and that Nrf2 levels were significantly reduced in urethral muscles in SUI rats compared with the controls. GS-Rb1 has been shown to modulate the expression and activity of numerous proteins and pathways, including the TGF-β1/Smad, PI3K/mTOR, Nrf2/ARE, and MAPK/NF-κB.
pathways[17]. A recent study showed that GS-Rb1 exerts cardioprotective effects against ischemia/reperfusion (I/R) or hypoxia/reoxygenation (H/R) injury[28]. Compression of the pelvic floor tissue during childbirth can cause ischemia and hypoxic injury of the pelvic floor tissue, leading to breakages in muscle and elastic fibers and reduced ECM. Our study found that the integrity of both muscle and elastic fibers in the GS-Rb1 groups was similar to that in the control group. We conclude that GS-Rb1 mitigates tissue damage through its antioxidant actions.

It has been reported that Nrf2 is an upstream modulator of TGF-β1/Smad3 in SUI rats[10]. GS-Rb1 thus acts as an antioxidant, alleviating oxidative damage through the upregulation of Nrf2/ARE. Here, GS-Rb1 was found to activate the expression of Nrf2 and ARE-containing genes. The urethral fibromuscular system was repaired in the SUI+GS-Rb1 groups. We thus conclude that GS-Rb1 may activate Nrf2/ARE to regulate the TGF-β1/Smad3 axis and initiate tissue repair. Our next study will investigate this topic in depth to obtain direct evidence for this hypothesis.

Conclusion

Ginsenoside GS-Rb1 was found to repair damage to the urethral fibromuscular system. GS-Rb1 was found to activate Nrf2/ARE, reducing oxidative damage to connective and muscle tissues. GS-Rb1 also appears to promote TGF-β1/Smad3 signaling to repair fiber damage and induce the synthesis of collagen I and collagen III. These findings suggest that Ginsenoside GS-Rb1 has the potential as an effective treatment for mechanical-trauma-induced SUI, providing a novel way of treating and preventing SUI.

Declarations

Funding Sources

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Statement of Ethics

This study protocol was reviewed and approved by Animal Experimental Ethics Committee of Shanxi Cancer Hospital (Approval Number:2021001, Shanxi, China). All animal procedures were performed by following the Guidelines for the Care and Use of Laboratory Animals. All experiments were reported in accordance with ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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Availability of data and materials

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Authors’ Contributions

All authors contributed to the study conception and design. Material preparation and data collection and analysis were performed by Shaohui Chen, Bingyan Wei, Sanyuan Zhang. The first draft of the manuscript was written by Shaohui Chen and Sanyuan Zhang put forward the main revision suggestion to the article, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References


**Figures**

**Figure 1**

Changes in urethral sphincter muscle structure and collagen concentrations.

First line: analysis of morphology of the urethral sphincter muscle structure by Masson's trichrome stain. Second line: magnification of the black rectangle in the first line. Control, SUI, SUI+L-Rb1, and SUI+H-Rb1 groups. Original magnification ×10 and ×40. Third line: Collagen contents of urethral and anterior vaginal wall tissue demonstrated by Picrosirius red staining. Fourth line: magnification of the black rectangle in the third line. Original magnification ×10 and ×20.
Figure 2

Elastic fibers of the urethra and vagina stained with Hart’s elastin stain.

Control (a–c), SUI (d–f), SUI+L-Rb1 (g–i), SUI+H-Rb1 (j–l). First column: analysis of morphology of the elastic fibers of the urethra and vagina. Original magnification ×20. Second column: magnification of the black rectangle in the first column; third column: magnification of the blue rectangle in the first column. Original magnification ×80.
Figure 3

(A) Real time RT-PCR was done using quantity one mRNA levels of TGF-β1, Smad3, Smad7, and collagens I and III in urethral and vaginal tissue. *P<0.05 **P<0.005. Control, SUI, SUI+L-Rb1, and SUI+H-Rb1 groups. (B) Protein bands showed the expressions of TGF-β1/Smad3 signaling pathway related proteins in urethra and anterior vaginal wall in all four groups. (C) Western blotting was performed to detect the protein expression of TGF-β1, Smad2, Smad3, Smad7, p-Smad3, p-Smad2, and collagens I and
III in the urethra and anterior vaginal wall of rats in four groups. The samples derive from the same experiment and that gels/blots were processed in parallel. Results are from three experiments.

**Figure 4**

Nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling related proteins expression in four groups. (A) Protein bands showed the expressions of Nrf2/ ARE signaling pathway
related proteins in urethra and anterior vaginal wall in all four groups. (B, C, D) Western blotting detected the protein expression of Nrf2, glutathione peroxidase (GPx1), and manganese superoxide dismutase (MnSOD) in the urethra and anterior vaginal wall of rats in four groups. *P<0.05 **P <0.005 vs SUI group. Results are from three experiments.

**Supplementary Files**

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